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Ricin Activity Assay by Direct Analysis in Real Time Mass Spectrometry Detection of Adenine Release

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Biotoxin activity assays typically involve multistep sample preparation, multicomponent reactions, multistep analysis, or a combination thereof. We report a single-step, real-time ricin activity assay that requires little or no sample preparation and employs direct analysis in real time mass spectrometry. The release of adenine from the inhomogeneous substrate herring sperm DNA by ricin was determined to be 53 ± 2 pmol adenine per picomole of ricin per hour. This procedure can be readily adapted to any enzyme for which a reactant or product of low molecular weight (up to ~600) can be identified.

Biological warfare agents include biological toxins, several of which are enzymes such as the botulinum toxins and ribosome-inactivating proteins (RIPs). Attacks using these toxins could be perpetrated by rogue groups or individuals and the results could be devastating. For example, 4 g of the RIP ricin, enough to kill 500 people, was illegally accumulated in a Las Vegas, Nevada, hotel room in 2008. Investigators believe the individual responsible was accidently poisoned with the ricin, causing a coma that lasted several weeks.[1] In 2004, powder containing ricin was found in a U.S. senate office building.[2] Once ricin was identified in the powder, the question became "How active [is it]?"[2] Until this question was answered the hazard assessment for the powder could not be completed. Because of its wide availability and ease of extraction from the castor bean, ricin remains a real danger to the public. These incidents highlight a very important point regarding analysis of unknown samples for biological toxins such as ricin: simple detection and identification of the toxin is only a first step in hazard assessment. Timely determination of the toxin's activity, the focus of this article, is also required.

Biotoxin activity assays typically involve multistep sample preparation, multicomponent reactions, multistep analysis, or a combination thereof. We report a single-step, real-time ricin activity assay that requires little or no sample preparation and employs direct analysis in real time (DART)[3] mass spectrometry (MS). Like the high-performance liquid chromatography (HPLC)−MS protocol reported by Hines et al.[4] involving only ricin, substrate, and internal standard, our approach does not require multiple components, cell cultures, coupled enzyme reactions, radioactive materials, or chemical modification of the substrate.[5−8] In addition, this procedure improves upon the HPLC−MS procedure in that no isotopic labeling of the internal standard is required, no introduction of ricin into the instrument occurs, and no quenching of the reaction occurs (i.e., monitoring is truly real-time). Furthermore, with little modification, the procedure could be applied to many possible enzymes.

Ricin is an enzyme of molecular weight above 60 000 composed of A- and B-chains of approximately equal molecular weights linked by a disulfide bridge. The B-chain binds to galactose moieties on the eukaryotic cell surface, thereby facilitating entry into the cell.[9] Once inside the cell, the A-chain catalyzes cleavage at adenosine 4324 (in rat RNA) of 28S rRNA to release adenine.[10] This action inhibits protein synthesis, leading to cell death. In addition to RNA, herring sperm DNA (hsDNA) is a substrate for ricin.[11] We chose to employ hsDNA for this assay because it is relatively stable and widely available. The procedure here applies direct analysis in real time mass spectrometry (DART-MS) to monitor adenine release from hsDNA.

The DART is an ion source used to analyze solids, liquids, gases, and solutions under ambient conditions.[3] Mass spectra are collected within a few seconds. It is therefore well suited to time critical analyses such as enzyme activity studies. Mass spectral

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interpretation is minimal because little or no fragmentation occurs and the DART has been used for MS analysis of a wide range of polar and nonpolar molecules including peptides, organometallics, and chemical warfare agents.[3,12,13] It has been employed without sample preparation steps for diverse sample matrixes such as pharmaceutical formulations and biological fluids.[3,12,13] While its use for enzymatic reactions has not been reported, it has been shown to be useful for monitoring simple organic reactions with few reaction byproducts.[13] DART is particularly suitable for reactions in which the reactant/product monitored is a low molecular weight amine up to molecular weight ~600. Adenine, at MW 135.13, is just such a compound.

EXPERIMENTAL SECTION

Reagents. Ricin (150 mM NaCl, 10 mM PO_4, 0.08% NaN_3, pH 7.8) was purchased from Vector Laboratories (Burlingame, CA) and the concentration determined (4.46 mg/mL) using an E_{280} of 1.434 L g^{-1} cm^{-1}.[14] The ricin M_r was ascertained based on nondenaturing sodium-dodecyl sulfate polyacrylamide gel electrophoresis using Kaleidoscope Prestained Standards and Tris-HCl gradient (4−20%) Ready Gels (Bio-Rad, Hercules, CA). The average M_r as determined from three gels was 65 000. Purified hsDNA (10 mg/mL) in DNase-free, RNase-free, distilled, deionized water was from Invitrogen (Carlsbad, CA). Adenine, cytosine, uracil, sodium acetate, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO).

Activity Assay. The hsDNA (200 μg) was heated in a Fisher Scientific dry bath incubator at 95 °C for 5 min to shear the DNA, followed by cooling on ice for another 5 min. The sheared DNA was diluted with 10 mM ammonium acetate pH 4.7 containing internal standard (uracil).[4] Ricin (5.80 μg) was added, and the reaction mixture was incubated at 37 °C. The total reaction volume was 100 μL (890 nM ricin, 2 mg/mL hsDNA). At regular intervals, a capillary tube was repeatedly dipped into the reaction solution and presented to the DART (3 times minimum per time point). For each time point, the mass spectra were recorded on a JEOL AccuTOF mass spectrometer, and the peak intensities from the repeat measurements were averaged.

Safety Considerations. Ricin is extremely toxic. Its use is controlled under the Biological Select Agents and Toxins program in the United States by the Centers for Disease Control and Prevention (Atlanta, GA). Handling of ricin should follow strict safety procedures determined in collaboration with the safety office of the research laboratory's organization.

RESULTS AND DISCUSSION

Determination of Internal Standard. In the presence of fixed concentrations of hsDNA and adenine, the responses of cystosine, thymine, and uracil as potential internal standards were recorded as DART source temperature and carrier gas flow rate were varied. Uracil gave the most intense signal with results the most similar to adenine in relation to parameter variation. Thus, uracil was employed as an internal standard for the remainder of experiments

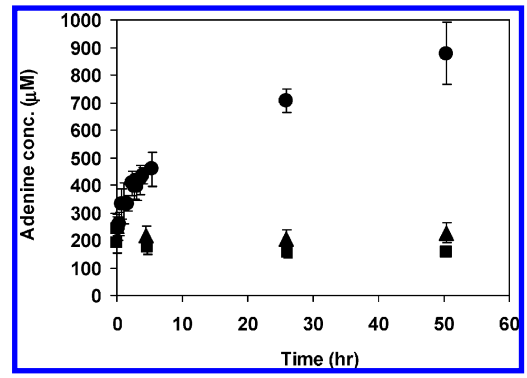

Figure 1. Adenine concentration over time. ● = reaction mixture containing 89 pmol of ricin with an initial concentration of 2 mg/mL hsDNA. ▲= reaction mixture containing 88 pmol BSA and hsDNA. ■ = blank reaction mixture containing only hsDNA.

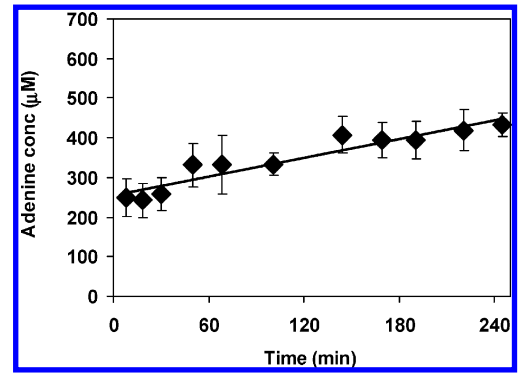

Figure 2. Adenine concentration over time for the initial 4 h in a reaction mixture containing 89 pmol of ricin and having an initial hsDNA concentration of 2 mg/mL.

(temperature = 180 °C, flow rate = 11.05 L/min). Temperatures above 200 °C resulted in hsDNA breakdown.

Linearity of Adenine Response. The response for adenine in the presence of 2 mg/mL hsDNA with uracil as an internal standard (892 μM) was linear over the range analyzed (2.9−740 μM, $R^2 = 0.986$). Herring sperm DNA produced a low-intensity interfering peak. The lowest adenine concentration (2.9 μM) evaluated could be readily detected above this peak with a signal-to-noise greater than 350.

Ricin Activity. Once linearity for adenine was ascertained, monitoring of adenine release from hsDNA by ricin was conducted. The reaction was followed for 50 h (Figure 1). The rate of adenine production was linear over the first 4 h and was determined to be 53 ± 2 pmol adenine/pmol of ricin/h (Figure 2). This value is on the same order as values of 70 pmol/pmol of ricin/h and 43 pmol/pmol of ricin A-chain/h obtained with hsDNA by Heisler et al. employing a colorimetric coupled enzyme assay[11] and to ~60 pmol/pmol of ricin A-chain/h obtained with a synthetic 14-mer RNA by Hines et al. employing LC−MS.[4] In comparison, the adenine concentration for hsDNA alone and for hsDNA in the presence of BSA (a nonenzymatic protein of similar molecular weight to ricin) was constant (Figure 1).

CONCLUSIONS

A new procedure using DART-MS for the determination of reaction rates of enzymes having low-molecular weight products or reactants has been demonstrated with the protein toxin ricin.

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The procedure can be employed to measure reaction rates of an enzyme in the presence of an inhomogeneous substrate such as hsDNA. While it is impossible to predict the character of all possible samples, this method, like many other DART methods,[12] is expected to be applicable to many complex or real-world matrixes. One complicating factor would be gross pre-existing sample contamination by adenine. In that event, a purification step may be necessary. The amount of ricin required (~90 pmol), on the order of 100 pmol rather than the 10 pmol required for the Hines et al.[4] procedure, remains well below a level toxic to humans. The advantage of a very simple, straightforward measurement procedure combined with the potential for direct continuous measurement under inhomogeneous conditions presents an attractive alternative to reported assays.

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